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ARTICLE

Correlation of Mycotoxin Fumonisin B_2 Production and Presence of the Fumonisin Biosynthetic Gene *fum8* in *Aspergillus niger* from Grape

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Aspergillus niger is a significant component of the fungal community on grapes. The mycotoxin fumonisin B_2 (FB_2) was recently detected in grape must and wine as well as in cultures of some *A. niger* strains isolated from grapes and raisins. This study examined 48 strains of *Aspergillus* section *Nigri* for the presence of the fumonisin biosynthetic gene *fum8* in relation to FB_2 production. The *fum8* gene was detected in only 11 *A. niger* strains, 9 of which also produced FB_2. Maximum parsimony analysis based on the calmodulin gene sequence indicated that the presence/absence of *fum8* is not correlated with the phylogenetic relationship of the isolates. This is the first report correlating the presence of a fumonisin biosynthetic gene with fumonisin production in *A. niger* from an important food crop. The results suggest that the absence of FB_2 production in grape isolates of *A. niger* can result from the absence of at least one gene essential for production.

KEYWORDS: Fumonisin; *Aspergillus niger*; *fum8* gene; grape

INTRODUCTION

Aspergillus section *Nigri* (black aspergilli) are reported as pre- and postharvest pathogens in bunch grapes, maize, cereal grains, onions, garlic, soybeans, apples, mangoes, and peanuts (*1*, *2*), although the extent of damage caused by this fungus to each host depends on unknown predisposing environmental factors. These fungi are an important food safety concern because they are prevalent on table and wine grapes and raisins from Mediterranean and South American countries as well as Australia (*4*) and some species can also produce the mycotoxins ochratoxins (OTA) and fumonisins (*3*−*8*). Moreover, some studies have shown that wines worldwide can be contaminated by fumonisins (*8*−*11*).

Fumonisins are polyketide-derived mycotoxins that cause multiple animal diseases, including cancer and neural tube defects in laboratory rodents (*12*). There are also epidemiological associations between consumption of fumonisin-contaminated maize and esophageal cancer and neural tube defects in some human populations for which maize is a dietary staple. Thus, the presence of fumonisins in grape products poses a further potential risk to human health.

Although fumonisin production by *Aspergillus niger* has been reported only relatively recently (*6*−*8*), production has been reported in species of the fungus *Fusarium* for over two decades (*12*). *Fusarium proliferatum* and *Fusarium verticillioides* are fumonisin-producing species of particular concern because of their widespread occurrence on maize. The predominant fumonisins produced by these species are FB_1, FB_2, and FB_3. These three fumonisin analogues differ in structure by the presence and absence of hydroxyl functions at C-5 and C-10. FB_1 has hydroxyl functions at both C-5 and C-10, FB_2 has a hydroxyl function at C-5 but not at C-10, and FB_3 has a hydroxyl function at C-10 but not at C-5 (*13*, *14*) (**Figure 1**).

Genetic and biochemical analyses of *F. verticillioides* have identified a fumonisin biosynthetic gene (*FUM*) cluster that consists of 17 genes (*15*, *16*). These *FUM* genes encode enzymes, transport proteins, and a transcription factor. The cluster genes *FUM1* and *FUM8* encode a polyketide synthase (Fum1p) and an α-oxoamine synthase (Fum8p), respectively, that are essential for synthesis of the 20-carbon-long chain that forms the backbone of FB_1, FB_2, and FB_3 (*13*, *17*, *18*). In the proposed fumonisin biosynthetic pathway, Fum1p catalyzes synthesis of a linear polyketide that forms carbon atoms 3 (C-3) through 20 (C-20) of the backbone as well as the methyl functions at C-12 and C-16. Subsequently, the Fum8p catalyzes condensation of the polyketide and the amino acid alanine to form C-1 and C-2 of the backbone as well as the amine function at C-2 (*16*−*18*). Other biosynthetic enzymes encoded by *FUM* cluster genes (e.g., *FUM2*, *FUM3*, *FUM6*, *FUM10*, and *FUM14*) are not required for synthesis of the backbone itself but instead catalyze addition of functional groups to various positions along the backbone or, in the case of *FUM13*, reduction of a functional group (*18*). Thus, in *F. verticillioides* inactivation of *FUM2*, *FUM3*, *FUM6*, *FUM10*, *FUM13*, or *FUM14* results in production of fumonisin biosynthetic intermediates or only FB_2 or FB_3 rather than the full complement of FB_1, FB_2, and FB_3. In contrast, inactivation of

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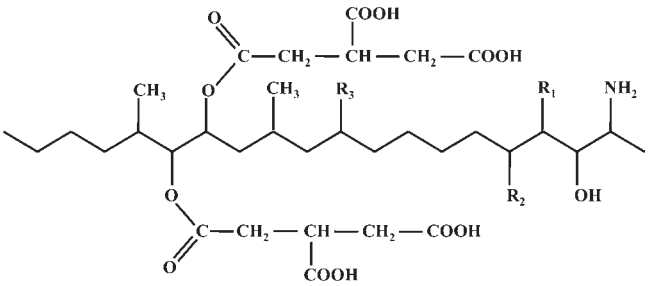

Compound	R_1	R_2	R_3
Fumonisin B_1	H	OH	OH
Fumonisin B_2	H	OH	H
Fumonisin B_3	OH	H	OH
Fumonisin B_4	H	H	H
Fumonisin B_6	OH	OH	H

Figure 1. Chemical structure of fumonisins and hydroxyl substitution positions.

either *FUM1* or *FUM8* blocks synthesis of the backbone and therefore production of all known fumonisin analogues and biosynthetic intermediates (*17*).

Recently, analysis of the genome sequence of *A. niger* revealed the presence of a putative fumonisin biosynthetic gene cluster in this fungus (*19*, *20*). The cluster includes orthologues of 10 of the genes in the *Fusarium FUM* cluster. Thus, seven genes that are in the *Fusarium* cluster are absent in the putative *A. niger* cluster. However, the region spanned by *FUM* gene orthologues in *A. niger* includes three genes that are not present in the *Fusarium* cluster. One of the genes present in the *Fusarium* cluster but absent in the *A. niger* cluster is *FUM2*. In *F. verticillioides*, *FUM2* encodes a monooxygenase that catalyzes hydroxylation of the fumonisin backbone at C-10 (*13*). Thus, the absence of *FUM2* in the *A. niger FUM* cluster is consistent with production in this species of FB_2, FB_4, and FB_6, which lack the C-10 hydroxyl, and the absence of production of FB_1 and FB_3, which have the C-10 hydroxyl (*7*) (**Figure 1**).

In previous analyses of *A. niger* isolates from grape, only 55% of the isolates produced FB_2; production was not detected in the remaining isolates examined (*9*). Variability in the presence or absence of production of a mycotoxin in a species has been reported among fumonisin and trichothecene-producing species of *Fusarium* and among aflatoxin-producing species of *A. parasiticus*. In the species *F. verticillioides*, which normally produces fumonisins, nonproducing isolates have fumonisin biosynthetic genes, but at least one of the genes is nonfunctional because of mutations within its coding region (*13*). In the current study, we examined grape isolates of *A. niger* for fumonisin production and the presence of an orthologue of *FUM8*, which served as a marker for the fumonisin biosynthetic gene cluster.

MATERIALS AND METHODS

Fungal Cultures and Genetic Nomenclature. Forty-eight fungal strains analyzed in this study were obtained from the ITEM culture collection at the Institute of Sciences of Food Production. Further information about the strains (year of isolation, depositor, toxin production, etc.) are available at the ISPA Website. The study included 32 strains of *A. niger* and one to four strains each of *Aspergillus brasiliensis*, *Aspergillus carbonarius*, *Aspergillus fetidus*, *Aspergillus japonicus*, *Aspergillus tubingensis*, and *Aspergillus uvarum*, species in section *Nigri* most closely related to *A. niger* (**Table 1**).

DNA Extraction. To prepare the DNA template for PCR, fungal strains were grown in shake cultures (120 rpm) in Wikerham's medium (40 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract, and water up to 1 L). About 15 mg of filtered, frozen, and lyophilized mycelium from each strain were used for total DNA extraction by NucleoMag 96 Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The quality of recovered DNA was assessed by gel electrophoresis and the quantity by Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE).

Table 1. FB_2 Production and DNA Analyses of Strains Tested in This Study

species	ITEM	FB_2 production[a] (μg/g)	PCR-*fum8*[b]	PCR-CL[c]	Southern *fum8*	Southern CL
A. niger	4501[T,d]	1.1	+	+	nt[e]	nt
	4502[T]	8.0	+	+	+	+
	4541	26.2	+	+	nt	nt
	4547	0.7	+	+	+	+
	4552	<DL	−	+	nt	nt
	4717	<DL	−	+	nt	nt
	4853	<DL	−	+	−	+
	4858	<DL	−	+	−	+
	4859	<DL	−	+	nt	nt
	4863	<DL	−	+	−	+
	4947	<DL	−	+	−	+
	4951	0.2	+	+	nt	nt
	5218	<DL	+	+	nt	nt
	5219	<DL	+	+	+	+
	5240	<DL	−	+	nt	nt
	5253	<DL	−	+	nt	nt
	5266	17.5	+	+	nt	nt
	5267	<DL	−	+	nt	nt
	5268	<DL	−	+	nt	nt
	5272	<DL	−	+	nt	nt
	5276	0.1	+	+	nt	nt
	5277	21.1	+	+	nt	nt
	5283	<DL	−	+	−	+
	6122	<DL	−	+	nt	nt
	6123	<DL	−	+	−	+
	6126	<DL	−	+	nt	nt
	6127	<DL	−	+	nt	nt
	6128	<DL	−	+	nt	nt
	6140	<DL	−	+	nt	nt
	6142	<DL	−	+	nt	nt
	6144	<DL	−	+	nt	nt
	7097	293	+	+	+	+
A. brasiliensis	4539	<DL	−	+	−	+
	4540	<DL	−	+	nt	nt
	6139	<DL	−	+	nt	nt
	7048[T]	<DL	−	+	nt	nt
A. carbonarius	4555	<DL	−	+	−	+
	4729	<DL	−	+	nt	nt
	4849	<DL	−	+	nt	nt
	5000	<DL	−	+	nt	nt
A. fetidus	4507	<DL	−	+	nt	nt
A. japonicus	4497	<DL	−	+	nt	nt
	7034	<DL	−	+	nt	nt
A. tubingensis	4720	<DL	−	+	-	+
	4721	<DL	−	+	nt	nt
	4948	<DL	−	+	nt	nt
	5017	<DL	−	+	nt	nt
A. uvarum	4844	<DL	−	+	nt	nt
F. verticillioides	7581	+	+	+	nt	nt

[a] Mean value of two replicates; detection limit = 1 ppb. [b] PCR with primers F1/R3 (this work). [c] PCR with primers CL1/CL2A (O'Donnell et al., 2000). [d T] = type-strain. [e] nt = not tested.

Table 2. Primers Designed in Conserved Region between *FvFUM8* and *Anfum8*

primer name	primer sequence (5′-3′)	*T* (°C)	position (bp) on AF155773.5 (*F. verticillioides*)	position (bp) on AM269971.1 (*A. niger*)
vnF1	TTCGTTTGAGTGGTGGCA	56	38073−38048	107745−107762
vnF2	CTGCAYTGGMGGATTYGTC	56	37986−37968	107832−107850
vnR1	TCAGAAWTAATSWCSMGTGGGA	56	37717−37696	108106−108127
vnR2	GCTACKGCAAAGCCCCT	57	37614−37598	108197−108213
vnR3	CAACTCCATASTTCWWGRRAGCCT	57	37187−37164	108584−108607

PCR Amplifications and Sequencing. Five PCR primers, vnNF1, vnF2, vnR1, vnR2, and vnR3 (**Table 2**), were designed for amplification of fragments of the *A. niger FUM8* orthologue (*Anfum8*). Primer design was based on conserved sequences of the *Anfum8* from *A. niger* strain CBS 513.88 (GenBank accession AM269971.1, locus An01g06870) and the *F. verticillioides FUM8* (*FvFUM8*) (GenBank accession AF155773.5). Previously described primers CL1 and CL2A were used for amplification of a ∼650 bp fragment of the calmodulin gene, *caM* (*21*), to determine phylogenetic relationships between species. For PCR amplification of *Anfum8*, total fungal DNA was used as a template in combination with five primer pairs, vnF1−vnR1, vnF1−vnR2, vnF1−vnR3, vnF2−vnR2, or vnF2−vnR3. The expected sizes of the products amplified with these primer combinations ranged from 382 to 863 bp. Amplification reactions were performed in a 25 μL volume: 0.6 U of Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 1× Taq Gold DNA polymerase buffer I, 300 mM of each primer, 200 mM of each deoxynucleoside triphosphate (Applied Biosystems), and approximately 10 ng of fungal DNA as template. Amplification conditions were as follows: denaturation at 94 °C for 10 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at 56 °C (both genes) for 50 s, extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C until recovery of the samples. After amplification, PCR products were electrophoresed in a 1.2% w/v EtBr-stained agarose gel with a 100 bp ladder (Gibco BRL, Grand Island, NY) run in a separate lane. Amplicons were purified by a Microcon-PCR filter unit (Millipore, Bedford, MA).

Sequence analysis of both strands of purified PCR products was performed with the Big-Dye Terminator Cycle Sequencing Ready Reaction Kit. Sequencing reactions were purified by filtration through Sephadex G-50 (Pharmacia, Uppsala, Sweden) equilibrated in double-distilled water and then analyzed with a 3730 DNA Analyzer (Applied Biosystems). Newly determined nucleotide sequences were deposited in the GenBank/EMBL/DDBJ database. Accession numbers are listed in **Table 3**.

Southern Blot Analyses. A subset of *Aspergillus* section *Nigri* strains was also examined for the presence of *Anfum8* and *caM* gene by Southern blot analysis. Genomic DNA was isolated from lyophilized mycelia that was ground to a powder and then suspended in extraction buffer (200 mM Tris-Cl, pH 8, 250 mM NaCl, 25 mM EDTA, pH 8, and 0.5% SDS) at 50 mg per 250 μL of buffer. Genomic DNA was purified from the suspension with the DNeasy Plant Mini Kit (Qiagen, Germantown, MD) following the protocol recommended by the manufacturer. Approximately 5 μg of genomic DNA was digested with restriction enzyme *Eco*RV, electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and then subjected to a standard Southern hybridization protocol. Hybridization probes corresponded to a ∼800 bp fragment of the *Anfum8* that was amplified by standard PCR methods with primers 1996 (5′-TCGTTTGAGTGGTGGCAGAATC-3′) and 1997 (5′-CCATACTTCTTGAAAGCCTCCT-3′) and a ∼650 bp fragment of the *caM* gene amplified with primers CL1 and CL2A (*21*). Both the *Anfum8* and *caM* gene fragments were amplified from genomic DNA prepared from *A. niger* strain ITEM 7097. Following amplification, DNA fragments were subjected to agarose gel electrophoresis, purified with the UltraClean (MoBio Laboratories) method, and then labeled with ^{32}P with the Ready-to Go DNA labeling kit (Amersham Biosciences).

Fumonisin Production in Culture. All *Aspergillus* section *Nigri* strains were examined for their ability to produce fumonisins by liquid chromatography coupled to TurboIonSpray ionization tandem mass spectrometry (LC-MS/MS). Fungal strains were grown on CY20S agar in darkness for 7 days according to the method of Frisvad et al. (*6*).

Sample Extraction. Toxin extraction was performed according to the method of Frisvad et al. (*6*) with slight modifications. One gram of agar culture was extracted with 5 mL of 70% (v/v) methanol. Samples were placed for 50 min in an ultrasonic bath and then filtered using RC 0.2-μm filters (Phenomenex, Torrance, CA). The extracts were dried using a centrifuge evaporator, dissolved in 5 mL of 70% methanol, and used for LC-MS/MS investigations. For recovery studies, 1 g of agar culture was spiked with FB_1 and FB_2 standards, dissolved in methanol, at two different concentrations (1 and 10 μg/g FBs per g of agar culture). Spiked cultures were placed at room temperature overnight and then extracted as described above. FB_1 and FB_2 recoveries in spiked samples averaged 81 and 72%, respectively.The detection limits (LOD) for FB_1 and FB_2 were 0.5 and 1 ppb, respectively. Quantification limits (LOQ) were 1 ppb for FB_1 and 2 ppb for FB_2.

Table 3. GenBank Accession Numbers of Representative Nucleotide Sequences Examined in This Study

	accession no.	
isolate (ITEM)	calmodulin	*FvFUM8*/*Anfum8*
4497	AJ582717	
4501	AJ964872	FN662673
4502	FN394678	FN662670
4507	AM419749	
4539	AM295179	
4540	AM295178	
4541	100% identity with FN394667	FN662672
4547	100% identity with AJ964872	FN662678
4552	FN394668	
4555	AJ582715	
4720	100% identity with AJ964876	
4844	100% identity with AM745754	
4951	FN394676	FN662668
5218	FN650330	FN662671
5219	100% identity with FN650330	FN662675
5266	100% identity with FN394678	FN662676
5267	100% identity with FN394670	
5268	FN394669	
5276	FN394673	FN662674
5277	100% identity with FN394668	FN662669
6139	AM295176	
7034	AJ964875	
7048	AJ280010	
7097	100% identity with FN394678	FN662677
7581	AF158315	100% identity with AF155773.5 (nt 37140−38064)

LC-MS/MS Methods. Chromatographic separation was performed using an HPLC apparatus equipped with two micropumps series 200 (Perkin-Elmer, Shelton, CT). The column used was a 150 × 2 mm i.d., 5 μm, Gemini Rp18 110 Å (Phenomenex).

The eluents were (A) water with 0.1% formic acid and (B) CH_3CN/MeOH (80:20 v/v) with 0.1% formic acid. The gradient program was as follows: 30−100% B over 8 min, 100% B for 3 min, 100−30% B over 3 min, at a constant flow of 0.2 mL/min. Injection volume was 20 μL.

MS/MS analyses of fumonisins were performed on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems) equipped with a TurboIonspray source. The declustering potential (DP) and the collision energy (CE) were optimized for each compound infusing directly into the mass spectrometer standard solutions (10 μg/mL) at a constant flow rate of 6 μL/min using a model 11 syringe pump (Harvard Apparatus, Holliston,

MA). Drying gas (air) was heated to 350 °C, and the capillary voltage (IS) was set to +5000 V. Analyses were performed in the positive ion mode in multiple reaction monitoring (MRM). A mass spectrometer declustering potential (DP) of 70 V gives precursor ion m/z 722.4 for FB_1, and the daughter ions are produced using CE at 50 and 42 V, giving m/z 352.4 and 528.1, respectively. Mass spectrometer conditions for FB_2 give with DP of 70 V a precursor ion at m/z 706.4, and the daughter ions are produced using CE at 51 and 42 V, giving m/z 336.2 and 512.2.

Phylogenetic Analysis. Sequences from a 700 bp fragment of the *caM* and an 800 bp fragment of *Anfum8* genes were used for the phylogenetic analyses. The DNA sequences were aligned using ClustalW with homologous sequences from *F. verticillioides*. Sequence identity was then confirmed using the GenBank BLASTn search. Phylogenetic trees were prepared by using the neighbor-joining method (*22*) in MEGA4 (*23*). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown (> 60%) above the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes−Cantor method and are in the units of the number of base substitutions per site.

RESULTS

PCR Screening for Anfum8. In this study, the presence of the fumonisin biosynthetic gene cluster in species of *Aspergillus* section *Nigri* was examined using the *Anfum8* gene as a genetic marker. This gene is an appropriate marker because it was previously shown to be present in fumonisin-producing *A. niger* strains ATCC 1015 and CBS 513.88 (*6*) and because the *F. verticillioides* orthologue (*FvFUM8*) is essential for fumonisin production (*17*).

In PCR experiments with the five degenerate *fum8*/*FUM8* primers (**Table 2**), only the vnF1−vnR3 primer combination consistently yielded a band of the expected size (~800 bp) from DNA templates prepared from the *Aspergillus* and *F. verticillioides* strains examined. All other primer combinations did not yield a product, yielded a product inconsistently, yielded a product that was not of the expected size, or yielded multiple products. Sequence analysis of the vnF1−vnR3 amplicon confirmed its identity as a fragment of *Anfum8*/*FvFUM8*. Among *Aspergillus* species, primers vnF1 and vnR3 amplified the *Anfum8* fragment from 11 of 32 *A. niger* strains tested (**Table 1**). These primers failed to amplify an *Anfum8* fragment from strains of the other 6 *Aspergillus* species examined (**Table 1**).

Southern Blot Analyses of *Anfum8* and *caM*. A subset of *Aspergillus* section *Nigri* strains was also examined for the presence of *Anfum8* by Southern blot analysis. The Southern and PCR analyses were consistent. Strains in which *Anfum8* was detected by Southern analysis were strains in which the gene was detected by PCR (**Figure 2A**). Likewise, strains in which the gene was not detected by Southern analysis were strains in which the gene was not detected by PCR. As a positive control, we also analyzed the DNA preparations by Southern analysis for the presence of the *caM* gene (**Figure 2B**). In contrast to *Anfum8*, *caM* was detected in all strains included in the Southern analysis.

Fumonisin Production. Among the 11 *A. niger* strains positive for the presence of *Anfum8*, 9 were also positive for FB_2 production at levels ranging from 0.1 to 293 μg/g (**Table 1**). Only 2 strains, ITEM 5218 and ITEM 5219, were positive for *Anfum8* and negative for FB_2 production, at least under conditions employed in this study. FB_2 production was not detected in the other 23 *A. niger* strains or in other *Aspergillus* species examined. To confirm the lack of FB_1 production by *A. niger*, all strains were analyzed also for FB_1. None of the strains tested was able to produce the mycotoxin, confirming the previous reports (*6*−*8*).

Phylogenetic Analysis. The genetic relationships of the *Aspergillus* strains included in this study were inferred by phylogenetic analysis of the nucleotide sequences of *Anfum8* and *caM*. The *Anfum8* analysis employed nucleotide sequences of the *Anfum8* fragment amplified with primers vnF1 and vnR3 from each of the 11 strains shown in **Figure 3**. In this analysis, *FvFUM8*, which shares 52% identity with *Anfum8*, was used as an outgroup. The analysis resolved the 11 *A. niger* strains into two distinct clades, designated here clade fum8-A and clade fum8-B. Clade fum8-A, which has a relatively low level of bootstrap support, includes strains ITEM 4502, ITEM 4541, ITEM 4951, ITEM 5218, ITEM 5219, ITEM 5266, ITEM 5277, and ITEM 7097, whereas clade fum8-B consisted of strains ITEM 4501, ITEM 4547, and ITEM 5276 (**Figure 3**). Between the two clades, *Anfum8* sequences were 96% identical and within each clade the sequences were 98−99% identical, respectively.

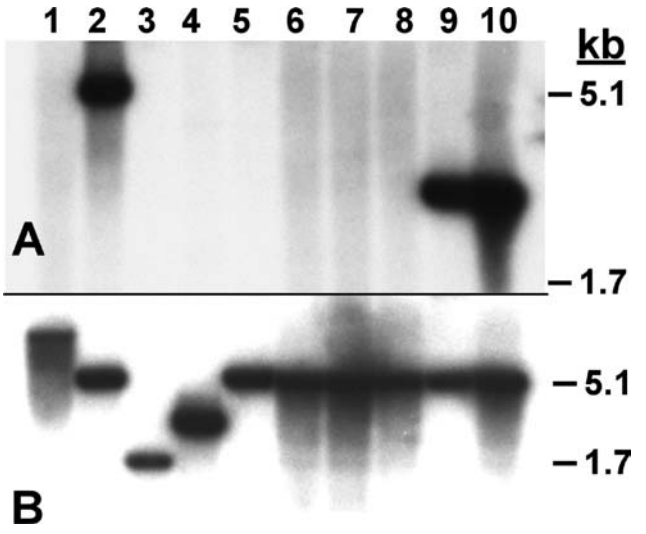

Figure 2. Southern blot analysis of *Anfum8* (**A**) and *caM* (**B**) genes in *Aspergillus* section *Nigri*. Lanes: 1, *A. brasiliensis* ITEM 4539; 3, *A. carbonarius* ITEM 4555; 4, *A. tubingensis* ITEM 4720; 2 and 5−10, *A. niger* ITEM 4547, ITEM 4853, ITEM 4858, ITEM 4863, ITEM 4947, ITEM 5219, and ITEM 7097, respectively. Numbers at right indicate positions of selected molecular weight markers (in kb).

The phylogenetic analysis with the *caM* sequence included all *Agillus* strains examined in the current study. This analysis resolved the *Aspergillus* strains into six distinct clades that corresponded to the six species examined, although *A. japonicus* and *A. uvarum* were resolved into the same clade, the uniseriate black aspergilli group, which they belong to (**Figure 4**). The 32 *A. niger* strains examined were resolved into two distinct clades, caM-A and caM-B, that were strongly supported by bootstrap values of 100 and 98, respectively (**Figure 4**). Clade caM-A included strains in which *Anfum8* was detected as well as strains in which the gene was not detected; in contrast, clade caM-B included only strains in which *Anfum8* was detected.

Comparison of the phylogenetic trees generated with the *Anfum8* and *caM* sequences revealed that positions of strains within the two trees were usually correlated. Indeed, the strains in clade fum8-A were the same as those in clade caM-A, and the strains in clade fum8-B were the same as those in clade caM-B. The exceptions to this were strains ITEM 5218 and ITEM 5219, which were resolved into clade fum8-A within the *Anfum8* tree and into clade caM-B within the *caM* tree (**Figures 3** and **4**). Thus, strains ITEM 5128 and ITEM 5219 were the only strains that grouped more closely with one set of strains in the *Anfum8* analysis but a different set of strains in the *caM* analysis.

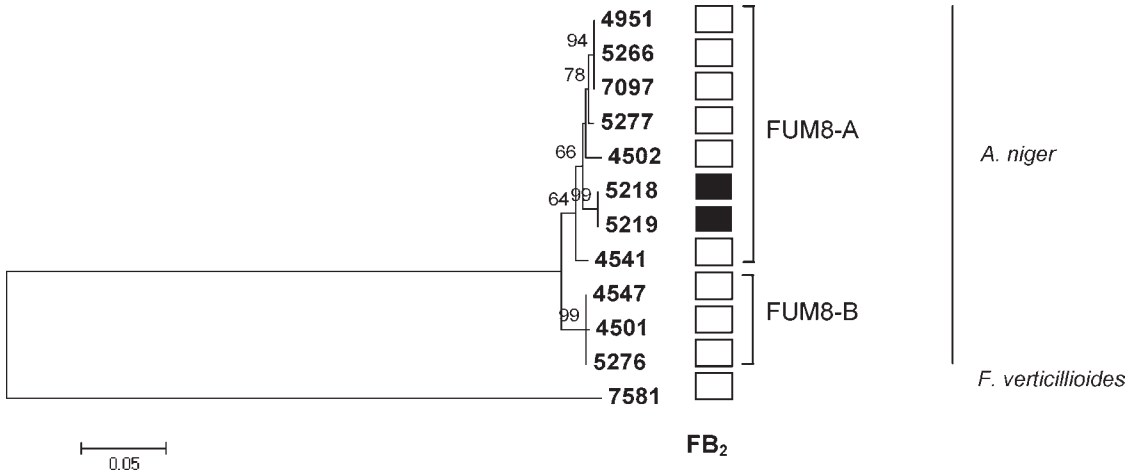

Figure 3. Evolutionary relationships of the *Anfum8* gene among 11 strains of *A. niger* isolated from grapes. The tree was generated from a ClustalW alignment of the nucleotide sequences of an 800 bp fragment of *Anfum8* amplified with primers vnF1 and vnR3. The optimal tree with the branch length sum of 0.74063002 is shown. There were a total of 649 positions in the final data set. Light squares indicate FB_2-producing strains and dark squares FB_2-nonproducing strains.

DISCUSSION

The results of this study represent the first investigation to correlate fumonisin production with the presence of a fumonisin biosynthetic gene in species of *Aspergillus* section *Nigri* that occur on grapes. The *Anfum8* gene was detected in strains of the species *A. niger* but not in strains of six other *Aspergillus* species examined. Within *A. niger*, *Anfum8* was detected in about one-third of the strains examined, and detection was correlated with the ability to produce FB_2. Thus, *Anfum8* was absent, or possibly highly divergent, in the majority of the fumonisin-nonproducing *A. niger* strains. It remains to be determined whether other fumonisin biosynthetic genes present in the published genome sequences of *A. niger* are absent or highly diverged in the fumonisin-nonproducing strains of *A. niger*.

Because many of the strains examined in this study were previously analyzed by AFLP (*23*), it was possible to compare the AFLP dendrogram and the phylogenetic tree generated from sequences of *Anfum8* and *caM* in this study. For the "*A. niger*-like" strains, the AFLP dendogram exhibited a topology similar to that of the *Anfum8*- and *caM*-based trees. In particular, ITEM 5218 and ITEM 5219 form a distinct clade with moderate to high bootstrap support in all three analyses.

Both the *caM*-based phylogenetic analysis and the AFLP analysis suggest that fumonisin-producing strains of *A. niger* can be as closely related to fumonisin-nonproducing strains as they are to other producing strains; fumonisin-producing and -nonproducing strains were present in the two major *A. niger* clades (caM-A and caM-B) resolved by phylogenetic analysis of the *caM* gene (**Figure 4**). Thus, fumonisin-producing and -nonproducing strains of *A. niger* do not appear to constitute genetically distinct subpopulations within the species. Likewise, within clade caM-A, strains with *Anfum8* could be as closely related to strains in which the gene was not detected as they were to other strains with *Anfum8*. Thus, the presence or absence of *Anfum8* was not associated with a more minor clade within clade caM-A. In contrast, *Anfum8* was detected in the five strains (ITEM 4501, ITEM 4547, ITEM 5218, ITEM 5219, and ITEM 5276) that formed clade caM-B (**Figure 4**). A clade corresponding to these same five strains was also resolved in the AFLP analysis reported by Perrone et al. (*24*). Thus, clade caM-B may represent a distinct subpopulation within *A. niger* in which the presence of *Anfum8* is continuous. If confirmed, this continuity contrasts with the discontinuity of the *Anfum8* in clade caM-A. How this discontinuity of *Anfum8* arose is not clear. It could have arisen by multiple independent losses of *Anfum8* or by a selection that has maintained the presence of the gene in some strains and its absence in others. In the *F. graminearum* species complex, there is evidence that balancing selection has maintained sequence polymorphisms within the gene cluster required for production of trichothecene mycotoxins (*25*). Thus, there is precedence for maintenance of sequence polymorphism in mycotoxin biosynthetic genes in other fungi (*26*). Several types of analyses have the potential to provide insight into the mechanism by which this *FUM* cluster polymorphism has been maintained in *A. niger*. First, the true nature of the polymorphism should be ascertained; that is, is the polymorphism due to the absence of *Anfum8* or to sequence divergence? Second, the extent of the polymorphism should be determined; that is, does polymorphism exist for other *A. niger FUM* cluster genes as well as genes flanking the cluster?

The lack of production of FB_2 in two *A. niger* strains (i.e., ITEM 5218 and ITEM 5219) in which *Anfum8* was detected is not surprising. There are numerous examples of strains of various species of fungi that have mycotoxin biosynthetic genes but that do not produce the corresponding mycotoxins (*13*, *27*). The absence of production can be due to a mutation in a biosynthetic gene, as in the case of an inactive *FUM1* gene within some fumonisin-nonproducing strains of *F. verticillioides* (*13*), or defects in gene expression, as occurred for aflatoxin biosynthetic genes in some strains of *Aspergillus* (*28*).

The apparent discontinuous distribution of *Anfum8* in *A. niger* is analogous, in some ways, to the absence of *FUM* cluster in isolates of *F. verticillioides* from banana and its presence in isolates from maize (*29*, *30*). However, the situations in *A. niger* and *F. verticillioides* are distinct when one considers the phylogenetic relationship of isolates with and without the *FUM* genes. Phylogenetic analyses indicate that banana isolates of *F. verticillioides* are distinct from and may be a different species (*F. musae*) from maize isolates of the fungus (*30*). In contrast, both the AFLP analysis (*24*) and *caM*-based phylogenetic analysis indicate that overall strains of *A. niger* that lack *Anfum8* do not represent a population that is distinct from those that have the gene.

In conclusion, our findings confirm the potential risk for FB_2 contamination of grapes and wine from *A. niger*. The results indicate that the absence of fumonisin production in the majority of the *A. niger* isolates is associated with the absence of at least

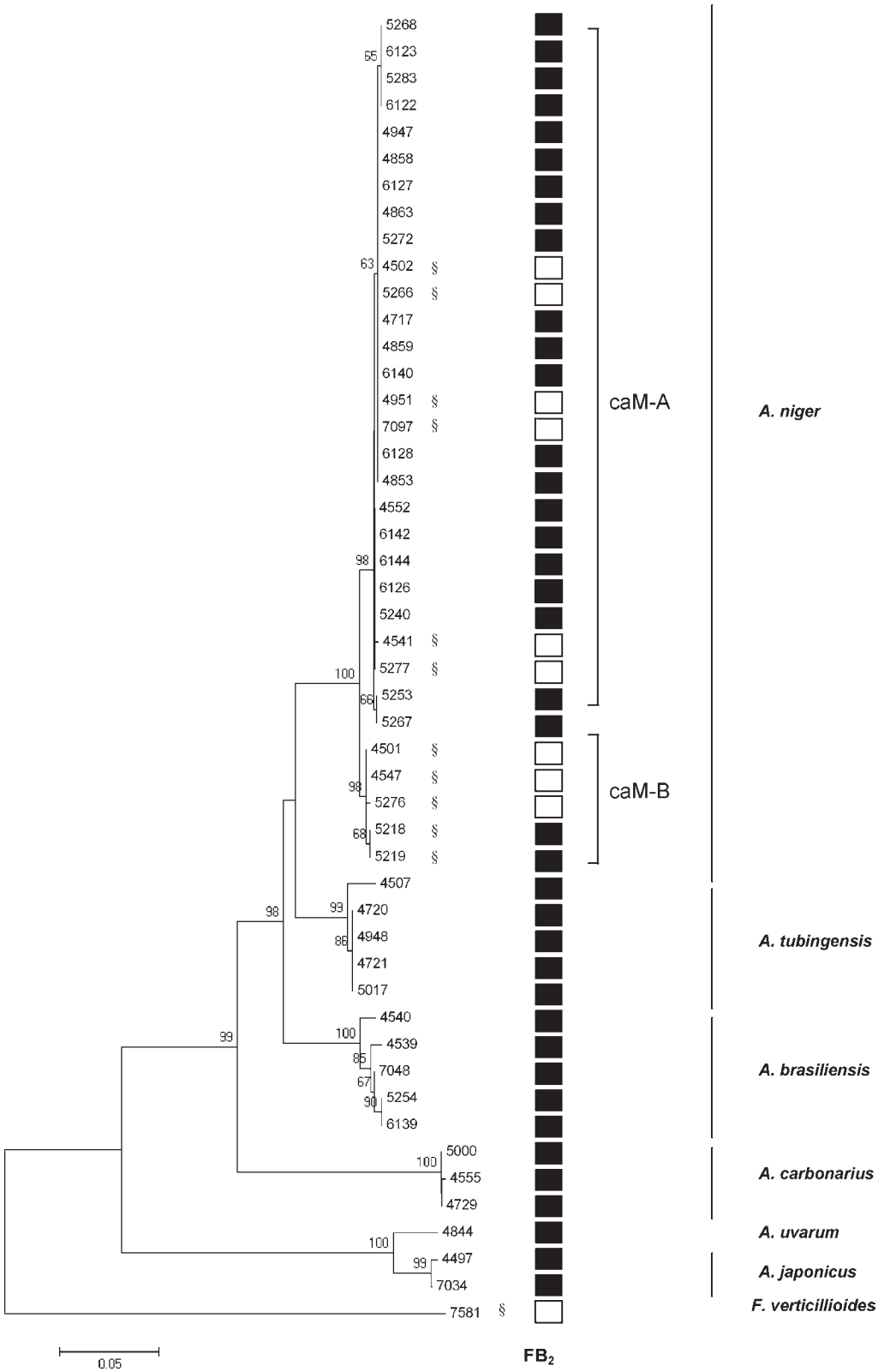

Figure 4. Evolutionary relationships of *caM* among 49 strains of *A. niger* isolated from grapes. The tree was generated from a ClustalW alignment of the nucleotide sequences of a 700 bp fragment of *caM* amplified with primers CL1 and CL2A. The optimal tree with the branch length sum of 0.81378311 is shown. There were a total of 552 positions in the final data set. Light squares indicate FB_2-producing strains and dark squares FB_2-nonproducing strains. § indicates *Anfum8* positive strains.

part of the fumonisin biosynthetic gene cluster. The discontinuous distribution of at least part of the fumonisin cluster in some strains also provides a possible explanation for the absence of fumonisin production in a large percentage of *A. niger* isolates and confirms the genetic complexity of this species. This in turn suggests that further analysis of the occurrence of the *FUM* cluster in *A. niger*, combined with wider phylogenetic analyses, would help to clarify the taxonomy of this complex species.

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